

LDN-73794 Attenuated LRRK2-Induced Degeneration in a *Drosophila* Parkinson's Disease Model

Dejun Yang^{1,2,3*}, Sharmila Das¹, Loujing Song¹, Tianxia Li¹, Jianqun Yan², Wanli W. Smith^{1*}

¹Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, USA

²Department of Physiology and Pathophysiology, Xi'an Jiaotong University School of Medicine, Xi'an, China

³Department of Neurology, University of Massachusetts Medical School, Worcester, USA

Email: *dejun.yang@umassmed.edu, *wsmith@rx.umaryland.edu

Received 10 June 2015; accepted 26 July 2015; published 29 July 2015

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Abstract

Parkinson's disease (PD) is a common neurodegenerative disease with unclear pathogenesis. Currently, there are no disease-modifying neuron-protecting drugs to slow down the neuronal degeneration. Mutations in the leucine-rich repeat kinase 2 (LRRK2) cause genetic forms of PD and contribute to sporadic PD as well. Disruption of LRRK2 kinase functions has become one of the potential mechanisms underlying disease-linked mutation-induced neuronal degeneration. To further characterize the pharmacological effects of a reported LRRK2 kinase inhibitor, LDN-73794, *in vitro* cell models and a LRRK2 *Drosophila* PD model were used. LDN-73794 reduced LRRK2 kinase activity *in vitro* and *in vivo*. Moreover, LDN-73794 increased survival, improved locomotor activity, and suppressed DA neuron loss in LRRK2 transgenic flies. These results suggest that inhibition of LRRK2 kinase activity can be a potential therapeutic strategy for PD intervention and LDN-73794 could be a potential lead compound for developing neuroprotective therapeutics.

Keywords

LRRK2, Parkinson's Disease, LDN-73794, Kinase Activity, Neuronal Degeneration, Dopamine Neuron, *Drosophila* Model

1. Introduction

Parkinson's disease (PD) is a common age-related progressive neurodegenerative disease with two pathological

*Corresponding authors.

How to cite this paper: Yang, D.J., Das, S., Song, L.J., Li, T.X., Yan, J.Q. and Smith, W.W. (2015) LDN-73794 Attenuated LRRK2-Induced Degeneration in a *Drosophila* Parkinson's Disease Model. *Advances in Parkinson's Disease*, 4, 49-58.
<http://dx.doi.org/10.4236/apd.2015.43007>

hallmarks: loss of dopaminergic neurons and the presence of proteinaceous aggregates called Lewy bodies. Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene has been identified to cause a genetic form of PD reported in 2004 [1] [2]. LRRK2 is expressed in the cytosol in neurons, microglia, and astrocytes in brains [3]-[6] and can be detected in Lewy bodies [7]. The physiological function of LRRK2 is not clear but it may play a role in neuronal outgrowth and guidance, as well as regulating gene expression [8]-[13].

LRRK2 contains a kinase domain and a GTPase domain with two enzymatic activities [1] [2] [14]-[16]. The physiological LRRK2 kinase substrate is not known. LRRK2 can undergo autophosphorylation and phosphorylate a generic substrate, myelin basic protein (MBP) [17] [18]. Moesin, 4E-BP, ezrine, radixin, and s15 have been reported as candidates for LRRK2 substrates [13] [19]-[21] although the physiological relevance of these proteins still requires further investigation. The majority of disease-linked LRRK2 mutations are in the GTPase domain or the kinase domain and alter the LRRK2 catalytic activity related to neuronal degeneration [12] [18] [22]-[24]. The most common PD mutation, G2019S-LRRK2, has been reported to increase kinase activity [17]-[19] [25] [26] and induce neuronal degeneration. Reduction of G2019S-LRRK2 kinase activity protects against neuronal degeneration [18] [23] [27]. Thus, inhibition of LRRK2 kinase activity may have therapeutic potential for PD intervention. Increasing efforts on developing LRRK2 kinase inhibitors lead to the discovery of several LRRK2 kinase inhibitors as “probes” to pharmacologically dissect the LRRK2 biological functions [23] [28]-[32]. Currently, most of the reported LRRK2 inhibitors, such as LDN-73794 [30], are in the early stages of evaluation using *in vitro* models. Liu *et al.* identified LDN-73794, which can inhibit LRRK2 with an IC_{50} of $3.5 \pm 0.3 \mu\text{M}$ by screen with a peptide (PLK-derived peptide with a motif of RRRSLLE) as a substrate [30]. LDN-73794 is a competitive inhibitor of the binding of ATP and a noncompetitive inhibitor of PLK-peptide. The *In vitro* studies showed that LDN-73794 inhibits LRRK2 kinase activity but has no effect on its GTPase activity. However, in pharmacological effects of LDN-73794 on LRRK2 in cells and *in vivo* models are not clear.

We recently developed a LRRK2 transgenic *Drosophila* model with severe locomotor impairment and robust DA neuron degeneration [34]. The human G2019S-LRRK2 is expressed under a dopaminergic neuron promoter in this model that becomes a useful *in vivo* animal model to test neuroprotective compounds targeting LRRK2 [28]. In this study, *in vitro* cell culture models and this LRRK2 *Drosophila* PD model were used to characterize the pharmacological effects of LDN-73794. Our results provided a proof of principle that LRRK2 kinase inhibition can be a novel therapeutic strategy for PD intervention and LDN-73794 can be a potential lead compound for further drug development.

2. Materials and Methods

2.1. Fly Stocks and Reagents

The *dopa decarboxylase* (ddc)-GAL4, UAS-LRRK2-1, and UAS-G2019S-2 fly stocks were maintained in our lab on standard fly food at 25°C as described previously [34].

Anti-tyrosine hydroxylase (TH) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-actin and anti-Flag antibodies were ordered from Sigma (St. Louis, MO, USA). LDN was purchased from Timtec. Fetal bovine serum, penicillin and streptomycin, LipofectAMINE Plus reagent and cell culture media were from Invitrogen (Carlsbad, CA).

2.2. Cell Culture and Transfection

Human HEK293T (human embryonic kidney) cells were from ATCC grown in DMEM with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin) at 37°C under 5% $\text{CO}_2/95\%$ air. The Flag tagged wild type LRRK2 and mutant G2019S constructs were transfected into HEK293T cells using Lipofectamine™ and PLUS™ Reagents (Invitrogen) followed the manufacturer’s protocol as described previously [18].

2.3. Mouse Primary Cortical Neuronal Cultures and Cell Toxicity Assay

Primary cortical neuronal cultures were from C57BL mice at embryonic day 15 as described previously [4]. Neurons were grown in the poly-D-lysine- and laminin-coated plates (BD Biosource, San Diego) using neurobasal medium with B-27 supplement, Glutamax, and antibiotics. To overcome the low transfection efficiency of

cultured neurons, various LRRK2 constructs were co-transfected along with GFP at 15:1 ratio into primary neurons by Lipofectamine 2000 according to the manufacturer protocol. After 48 h transfection, neurons were fixed with 4% paraformaldehyde and subjected to immunostaining using anti-Flag antibodies followed by 0.8 $\mu\text{g}/\text{mL}$ of bisbenzamide (Hoechst 33,342, Sigma) labeling. Most of the GFP positive cells up to 95% expressed LRRK2 as described previously [4] [28]. Cell images were taken from 40 fields in each experimental group. The healthy GFP-positive cells were automatically counted under a fluorescence microscope (Axiovert 200, Zeiss). Cell viability was measured as described previously [28], only the GFP positive cells were quantified.

2.4. Immunoprecipitation (IP), Western Blot, and Autophosphorylation (Kinase) Assays

Cell lysates or fly head homogenates were subjected to IP using anti-FLAG-agarose (Sigma) as described previously [4]. For western blot analysis, 4% - 12% NuPAGE Bis-Tris gels were used to separate the proteins that were then transferred into polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were probed with various primary antibodies followed by incubation with secondary detection antibodies. Proteins were detected by enhanced chemiluminescence (ECL) reagents. *In vitro* autophosphorylation (kinase) assay was described previously [18]. Briefly, kinase reactions were performed in kinase assay buffer with 500 μM ATP, 10 μCi of [γ - ^{32}P] ATP (3000 Ci/mmol) and 50 mM MgCl_2 for 90 min. The reaction was stopped by adding Laemmli sample buffer, and boiled for 5 min. The samples were subjected to 4% - 12% SDS/PAGE and blotted onto PVDF membranes. The membranes were quantified using a phosphoimager (Bio-Rad Molecular Imager FX). For some experiments, LRRK2 autophosphorylation was performed using anti-Flag IP followed by western blot analysis using anti-phosphorylated LRRK2 antibody as described previously [33] [35].

2.5. Survival and Climbing Assay

Cohorts of 60 flies from each experimental group were subjected to survival counting weekly. Vehicle and LDN-73794 was added into fly food from day 1 of eclosion throughout the fly lifetime. Fresh food with LDN-73794 was changed every 3 days. Mortality was analyzed by using Kaplan-Meier survival analysis. A p value < 0.05 was considered significant. Climbing assays (negative geotaxis assay) were employed to determine locomotor activity weekly as described previously [34]. Briefly, flies were placed in a vertical plastic cylinder (diameter, 1.5 cm; length, 25 cm) and tapped to the bottom. There were 60 flies from each experimental group. The flies that could climb to or above the median line of the cylinder within 10 seconds were counted.

2.6. Whole-Mount Brain Immunostaining and Dopamine (DA) Neuron Quantification

Adult fly brains at 6 weeks of age were dissected and subjected to whole-mount immunostaining using anti-TH antibodies as described previously [34]. Cohorts of eight flies per group were subjected to immunostaining with mouse monoclonal anti-TH (Immunostar) or rabbit polyclonal anti-TH (Chemicon) antibodies as the primary antibodies. Alexa Fluor 568 or Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) were used as secondary antibodies. The number of the neurons in paired anterolateral medial (PAM) results in high fluorescent density and cannot be quantified precisely. Except PAM, the numbers of TH-positive neurons in all other DA clusters were counted under fluorescent microscopy (Zeiss LSM 250).

2.7. Data Analysis

Quantitative data were expressed as arithmetic means \pm SEM based on at least three separate experiments. Statistically significant differences among groups were analyzed by ANOVA using Sigstart 3.1 statistical software (Aspire Software International, VA). A p value < 0.05 was considered significant.

3. Results

3.1. LDN-73794 Reduced LRRK2 Toxicity in Primary Neurons

To further characterize the effects of LDN-73794 [30], HEK 293T cells were used to study its effect on LRRK2 autophosphorylation. Cells were transfected with Flag-LRRK2 for 36 hours and serum was withdrawn for an additional 10 hours, followed by addition of LDN-73794 at a 50 μM concentration for 1 hour. Cell lysates were subjected to an *in vitro* LRRK2 phosphorylation assay. LDN-73794 reduced LRRK2 autophosphorylation at the

Ser2032 residue up to 82% compared with vehicle treated control cells (**Figure 1(b)** and **Figure 1(c)**). This is consistent with the previously findings that LDN-73794 reduced LRRK2 kinase activity using a PLK-peptide as a substrate [30].

To assess the effect of LDN-73794 on LRRK2 induced cell toxicity, mouse primary cortical neurons at 7 days *in vitro* were co-transfected with wild type LRRK2 or G2019S-LRRK2 and GFP at a 15:1 ratio and treated with LDN-73794 for 48 h. LDN-73794 reduced LRRK2-induced neuron toxicity compared with vehicle treated cells expressing GFP/G2019S-LRRK2 (**Figure 1(d)**) at a dose dependent manner (data not shown). 5 μ M LDN-73794 significantly attenuated LRRK2-induced neural loss, and have no effects on the survival of cells expressing GFP and control vector.

3.2. LDN-73794 Increased Survival and Improved Locomotor Impairment in G2019S-LRRK2 Transgenic Flies

The *UAS-WT-LRRK2* and *UAS-G2019S-LRRK2* flies crossed with the *ddc-GAL4* driver flies resulted in LRRK2 variant expression in DA neurons as described previously [34]. LDN-73794 (5 μ M) was added to fly food from day 1 post-eclosion throughout lifetime. The life span of non-pathogenic control flies (*UAS-LRRK2* or *ddc-GAL4* flies) were about 12 - 13 weeks [34]. Flies expressing wild type and G2019S-LRRK2 significantly reduced survival rate (**Figure 2**) as consistent with previous findings [34]. LDN-73794 did not alter the lifespan of non-pathogenic control flies (**Figure 2**). However, LDN-73794 significantly increased the survival of *ddc-GAL4*;LRRK2 and *ddc*:GAL4;G2019S flies compared with vehicle treated transgenic flies (**Figure 2(a)** and **Figure 2(b)**). Flies expressing wild type or G2019S-LRRK2 in DA neurons displayed severe locomotor impairment as previously described [34]. LDN-73794 did not alter the locomotor activity of non-pathogenic flies (**Figure 3(a)** and **Figure 3(b)**). Treatment with LDN73794 significantly improved the locomotor impairment of LRRK2 and G2019S transgenic flies compared with untreated transgenic flies (**Figure 3(a)** and **Figure 3(b)**).

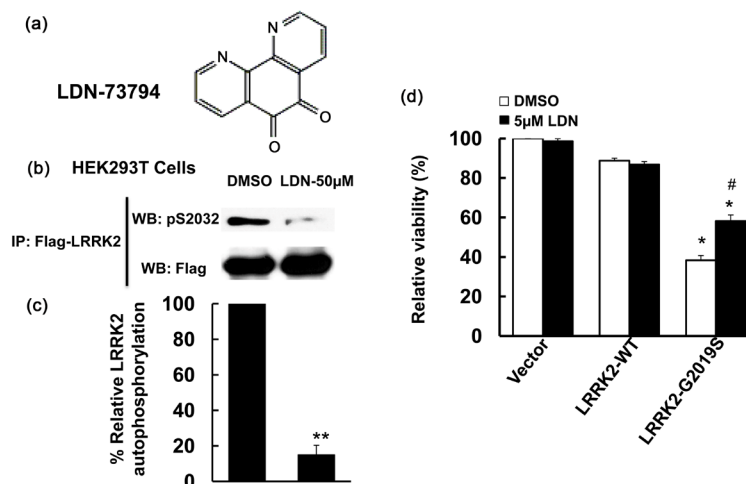


Figure 1. LDN-73794 reduced LRRK2 phosphorylation and protected against LRRK2 toxicity. (a) Chemical structure of LDN-73794 [31]; (b) LDN-73794 reduced LRRK2 autophosphorylation. HEK293T cells were transfected with Flag-LRRK2-G2019S for 36 h and followed by serum withdrawal for 10 h, then treated with LDN-73794 (0 and 50 μ M) for 1 h. Cell lysates were subjected to immunoprecipitation using anti-Flag antibodies and followed by western blot analysis using anti-phospho-LRRK2 antibodies at Ser2032 residue. Shown are representative images from three independent experiments; (c) Quantitation of B. ** $p < 0.01$ by ANOVA; (d) LDN-73794 attenuated LRRK2-induced neuronal degeneration in mouse cortical primary neurons. Mouse primary cortical neurons were co-transfected pCDNA-GFP with LRRK2-WT or LRRK2-G2019S at 1:15 ratio. After 4 h transfection, cells were treated with LDN-73794 for 44 h. The average intensity of DAPI stained nuclei of transfected cells were measured for cell viability assays as described previously [28]. Data are means \pm s.d. for three separated experiments, * $p < 0.05$ by ANOVA, vs vehicle or LDN-73794 treated groups. # $p < 0.05$ by ANOVA, vs untreated cells expressing LRRK2 or LRRK2-G2019S.

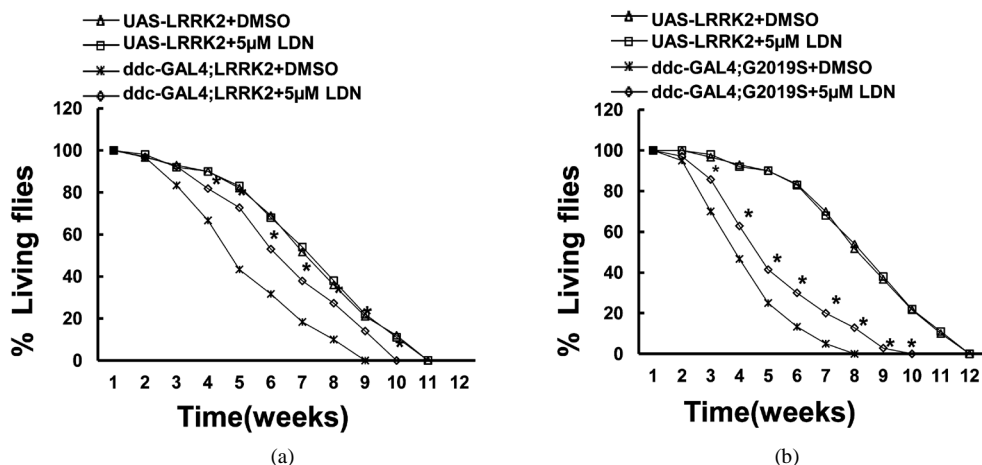


Figure 2. LDN-73794 increased survival of LRRK2 transgenic flies. The ddc-GAL4; LRRK2 (a) or ddc-GAL4; G2019S (b) flies were left untreated or treated with 5 μ M LDN-73794 from day 1 post-eclosion throughout life-time. Cohorts of 60 flies were in each experimental group. Survival data were analyzed by Kaplan-Meier log Rank survival analysis. $p < 0.05$, statistically significant differences between non-pathogenic and LRRK2 transgenic lines; between vehicle and LDN-73794 treated transgenic flies.

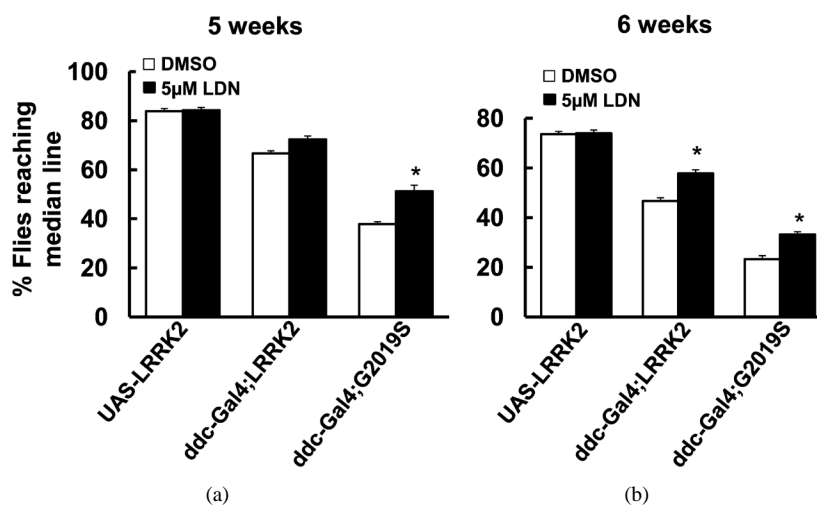


Figure 3. LDN-73794 improved locomotor impairment of G2019S-LRRK2 transgenic flies. The ddc-GAL4; LRRK2 or ddc-GAL4; G2019S flies were left untreated or treated with 5 μ M LDN-73794 from day 1 post-eclosion until 6 weeks of age. Cohorts of 60 flies (5 - 6 weeks of age) from each experimental group were subjected to climbing assays. (a) The climb assay result at 5 weeks; (b) The climb assay result at 6 weeks. Values are mean \pm s.e.m. *: $p < 0.05$ by ANOVA, vs vehicle treated LRRK2 transgenic flies.

3.3. LDN-73794 Suppressed DA Neuron Loss in G2019S-LRRK2 Transgenic Flies

To assess the effect of LDN-73794 on LRRK2-induced DA neuron degeneration in flies, whole-mount brain immunostaining was employed to detect the DA neurons using anti-TH antibodies. Expression of both wild type and G2019S-LRRK2 reduced the TH-positive neurons compared with non-pathogenic flies (Figure 4), consistent with previous reports [34]. Mutant G2019S transgenic flies resulted in severe loss of TH-positive neurons. Treatment with LDN-73794 suppressed G2019S-LRRK2-induced loss of DA neurons in all clusters (Figure 4). LDN-73794 significantly improved the TH-positive neuron loss in the PPM1/2 clusters (Figure 4(b) and Figure 4(c)). To further assess the effects of LDN-73794 *in vivo*, flies expressing mutant G2019S-LRRK2 were treated LDN-73794 for 2 weeks. Fly head homogenates were subjected to phosphorylation and kinase activity assays. LDN-73794 significantly attenuated LRRK2 phosphorylation in both wild type and G2019S-LRRK2 flies compared with vehicle treated transgenic flies (Figure 5).

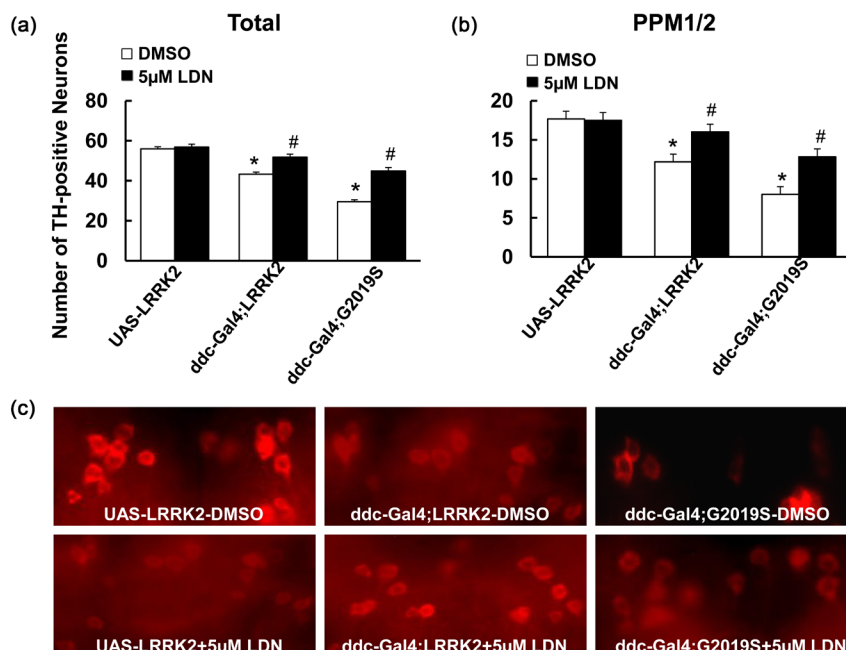


Figure 4. LDN-73794 attenuated LRRK2-induced DA neuron loss. Flies were left untreated or treated with 5 µM LDN-73794 from day 1 post-eclosion until 6 weeks of age. The fly brains were dissected at 6 weeks of age and subjected to immunostaining using anti-TH antibodies. The DA neurons were counted in all clusters except PAM. (a) Quantification of total DA neurons in all clusters except PAM; (b) Quantification of TH-positive neurons in PPM1/2 clusters; (c) Representative images of DA neurons in PPM1 and PPM2 clusters at 6 weeks of age. **p* < 0.05 by ANOVA, vs non-transgenic flies; #*p* < 0.05 by ANOVA, vs vehicle treated LRRK2 transgenic flies.

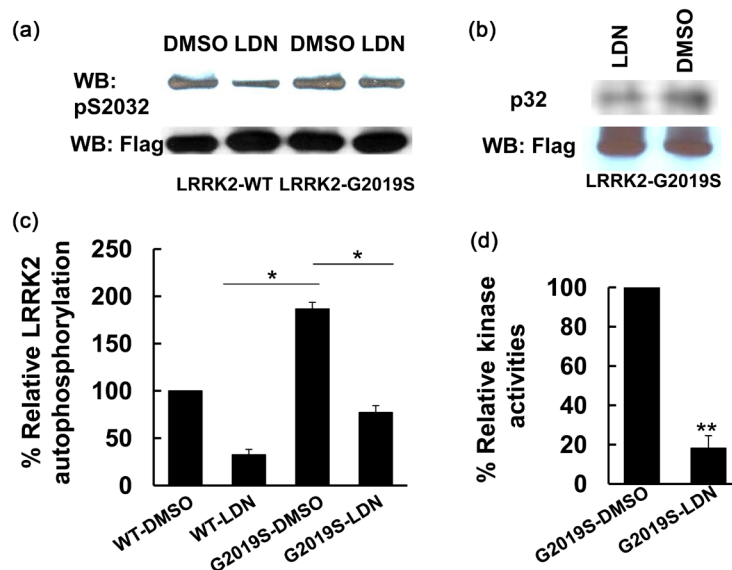


Figure 5. LDN-73794 reduced LRRK2 kinase activity in transgenic flies. G2019S-LRRK2 transgenic flies were left untreated or treated with 5 µM LDN-73794 for 2 weeks. Fly head homogenates were subjected to LRRK2 phosphorylation ((a) and (c)) or LRRK2 *in vitro* kinase ((b) and (d)) assays. (a) and (c), Fly head homogenates were immunoprecipitated using anti-Flag antibodies. The resulting immunoprecipitates were subjected to Western blot analysis using anti-phosphorylated LRRK2 antibodies against S2032 residue. (a) shown are representative blots from three independent experiments; (c) The quantitation of (a). Values are mean ± s.e.m. **p* < 0.05 by ANOVA as indicated; (b) and (d). Fly head homogenates were subjected to *in vitro* LRRK2 kinase assays using p32 incorporation as described previously [28] [34]; (b) Shown are representative image from three independent kinase assays; (d) The quantitation of (b), ***p* < 0.01 by ANOVA.

4. Discussion

In this study, characterization of a LRRK2 kinase inhibitor, LDN-73794, demonstrated that it reduced LRRK2 autophosphorylation at Ser2032 in HEK293T cells and protected against mutant G2019S-LRRK2 toxicity in mouse primary neurons. Moreover, LDN-73794 significantly increased survival, improved locomotor activity, and suppressed DA neuron loss in a LRRK2 *Drosophila* model. LDN-73794 also reduced G2019S-LRRK2 phosphorylation in brains of transgenic flies. These results suggest that inhibition of LRRK2 kinase activity can be a potential therapeutic strategy for PD intervention and LDN-73794 could be a potential lead compound for developing novel therapeutics.

LRRK2 kinase activity is altered by several disease-linked LRRK2 mutations in the GTPase or kinase domain including R1441C, R1441H, R1441G, Y1699C, G2019S, and I2020T [12] [18] [36]. The most common PD-linked mutation, G2019S, contributes to about 40% of genetic PD cases and some of the sporadic PD cases as well [37]. G2019S consistently increased LRRK2 protein kinase activity and caused severe neuronal degeneration in cultured neurons [4] [18] [24] [38]. Genetic alterations reducing kinase activity suppress LRRK2-toxicity [18] [25]. Our data showed that LDN-73794 could cross the cell membrane and inhibit LRRK2 phosphorylation in HEK293T cells expressing recombinant LRRK2 proteins. Treatment of LDN-73794 in primary neurons expressing mutant LRRK2 significantly reduced the neuronal degeneration. These results are consistent with recent findings that inhibition of LRRK2 kinase activity by the Raf kinase inhibitors, GW 5074 and sorafenib, displays neuron protective effects against LRRK2 toxicity [22] [28] [38].

DA neuronal degeneration is the early event in PD pathogenesis. To date, there are still no neuron-protecting drugs for PD treatment. One of the barriers to developing protective compounds is that rodent *in vivo* genetic testing models require a very long experimental time period. Moreover, LRRK2 transgenic rodent models [36] lack DA neuron degeneration or only display subtle degeneration, which hinders the drug testing [36].

Our *Drosophila* PD model expressing human mutant G2019S-LRRK2 provides the unique whole animal testing system for identifying neuronal protection compounds [34]. Unlike LRRK2 transgenic mouse models [36], flies expressing mutant LRRK2 displays severe motor dysfunction and selective DA neuron degeneration [34]. The *Drosophila* model provides a critical testing tool linked between *in vitro* model testing and clinical trials for developing PD-related compounds. In current study, we found that LDN-73794 significantly suppressed LRRK2-induced toxicity in cell and *Drosophila* models via reducing LRRK2 kinase activity. Although more detailed characterization (including the potential side effects) in animal models are remain to be examined, the Our findings provided the *in vivo* evidence that LDN-73794 inhibits LRRK2's kinase activity and suppresses the PD-like phenotype that can become the starting point for drug development to treat PD.

Acknowledgements

We thank Dr. Ted D. Dawson for providing the anti-phosphorylated LRRK2 antibodies.

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Abbreviations

PD, Parkinson Disease;
LRRK2, Leucine-Rich Repeat Kinase 2;
TH, Tyrosine Hydroxylase;
PAL, Protocerebral Antero Lateral;
PAM, Paired Anterolateral Medial;
PPM, Protocerebral Posterior Medial;
DA, Dopamine;
IP, Immunoprecipitation;
ddc, The *Dopa Decarboxylase*.